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Award Number: DAMD17-01-1-0290

TITLE: Role of CYP1B1 in PAH-DNA Adduct Formation and Breast Cancer Risk

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REPORT DATE: September 2004

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
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20050315 011

**REPORT DOCUMENTATION PAGE**Form Approved  
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

<b>1. AGENCY USE ONLY</b> (Leave blank)		<b>2. REPORT DATE</b> September 2004		<b>3. REPORT TYPE AND DATES COVERED</b> Annual (1 Sep 2003 - 31 Aug 2004)	
<b>4. TITLE AND SUBTITLE</b> Role of CYP1B1 in PAH-DNA Adduct Formation and Breast Cancer Risk				<b>5. FUNDING NUMBERS</b> DAMD17-01-1-0290	
<b>6. AUTHOR(S)</b>  Regine Goth-Goldstein, Ph.D.					
<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b> Ernest Orlando Lawrence Berkeley National Laboratory Berkeley, CA 94701  E-Mail: r_goth-goldstein@lbl.gov				<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>	
<b>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b> U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				<b>10. SPONSORING / MONITORING AGENCY REPORT NUMBER</b>	
<b>11. SUPPLEMENTARY NOTES</b>					
<b>12a. DISTRIBUTION / AVAILABILITY STATEMENT</b> Approved for Public Release; Distribution Unlimited				<b>12b. DISTRIBUTION CODE</b>	
<b>13. ABSTRACT (Maximum 200 Words)</b>  The cytochrome P450 enzyme CYP1B1 is a major enzyme involved in metabolizing polycyclic aromatic hydrocarbons (PAHs) to reactive intermediates. Given that certain exposure to PAHs may be a breast cancer risk factor, we have set out to examine CYP1B1 gene expression in breast tissue. After considerable delays in obtaining approval from the Brazilian National Ethics Committee for our protocol for human subject use the project is now operational. A laboratory has been set up in Porto Alegre. The questionnaire and protocols for specimen collection and processing have been finalized. The newly hired Research Coordinators and interviewers have been trained and recruitment of participants has begun.  The method to measure CYP1B1 expression has been improved by developing a quantitative RT-PCR assay using a quantitation standard. This quantitation standard was produced by cloning the CYP1B1 PCR fragment into a vector and a dilution series of this standard will be used in each experiment to quantify the absolute amount of CYP1B1 transcripts.  Because of the delayed start of the project, we have applied for and obtained a no cost extension.					
<b>14. SUBJECT TERMS</b> xenobiotic metabolism, CYP1B1, gene expression, genetic polymorphism, DNA adducts				<b>15. NUMBER OF PAGES</b> 25	
				<b>16. PRICE CODE</b>	
<b>17. SECURITY CLASSIFICATION OF REPORT</b> Unclassified	<b>18. SECURITY CLASSIFICATION OF THIS PAGE</b> Unclassified	<b>19. SECURITY CLASSIFICATION OF ABSTRACT</b> Unclassified	<b>20. LIMITATION OF ABSTRACT</b> Unlimited		

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)  
Prescribed by ANSI Std. Z39-18  
298-102

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## INTRODUCTION

This study investigates underlying molecular mechanisms of the relationship between PAH exposure and breast cancer risk. In breast tissue, the cytochrome P450 enzyme CYP1B1 appears to be a major enzyme involved in metabolizing PAHs to reactive intermediates (Goth-Goldstein et al., 2003). High CYP1B1 enzyme levels may result in increased formation of PAH-DNA adducts in breast tissue, subsequently leading to development of breast cancer (Lagueux et al., 1999). Gene expression analysis captures the convergence of multiple genetic and environmental factors that influence metabolic enzyme levels (Gonzalez and Gelboin, 1994; Whitlock, 1999). Using a clinic-based case-control design, breast tissue will be obtained from female patients undergoing either mastectomy or reduction mammoplasty surgery in Porto Alegre, Brazil. PAH exposure and potential confounding factor data will be collected for all cases and controls via medical chart review and an interviewer-administered questionnaire. To characterize molecular level interindividual variation in PAH metabolism, *CYP1B1* gene expression and PAH-DNA adducts will be measured in the surgically obtained breast epithelial cells from at least 37 cases and 74 controls undergoing mastectomy and reduction mammoplasty surgery, respectively.

## BODY

### ***Task 1: Identify study participants undergoing reduction mammoplasties or mastectomies and collect data and tissue –in progress***

While awaiting approval from the Brazilian National Ethics Committee (CONEP), we hired two part-time Research Coordinators to oversee day-to-day operations of the study, to coordinate interviews, collect and process biologic specimen, store and transport specimens, review and abstract information from participants' medical records, and manage data and study records. In addition, three interviewers and a database consultant were hired. Supplies for the project were brought to Porto Alegre, Brazil and a laboratory set up in Porto Alegre. Protocols for collection and processing of breast tissue, blood and urine were finalized (see Attachments). Recruitment Form listing eligibility criteria, Participant Tracking Form and Medical abstraction form were developed. The questionnaire was finalized and translated into Portuguese, back translated to English, and revised until both translators were satisfied with the questionnaire wording. Using these various protocols the Research Coordinators and interviewers were trained. Also, an Interviewer Manual and Data Entry System (protocol for data entry and codebook) are being developed. To monitor progress in participant recruitment and specimen collection we have established monthly conference calls with Brazilian field staff and monthly reports.

After receiving approval from CONEP in March of this year, the project is now fully operational. The revised questionnaire has been tested and participant recruitment is in progress. However, enrollment of participants has been slower than anticipated. Recent changes of standard of care indicate that chemotherapy be administered before surgery for some of those undergoing mastectomy surgery – this conflicts with our eligibility criteria of no prior chemotherapy. Therefore, we are now collecting tissue from women undergoing partial mastectomy. We have revised the Human Subjects Protocol to include patients being seen by any surgeon in Dr.

Caleffi's group and we are awaiting approval of these revisions by the University of California Office for Protection of Human Subjects. At this time, we have enrolled 5 participants (4 cases, 1 control). Specimens will be shipped to LBNL on September 28, 2004. We established conditions for proper shipping of specimens. Originally it had been planned that one of the investigators would hand-carry specimens to the United States. However, new regulations do not allow this and specimens will be shipped through World Courier.

**Task 2: Characterize the breast tissue samples in respect to *CYP1B1* expression and *CYP1B1* polymorphism – preliminary experiments**

Specimens collected in this study will be a complex mixture of varying cell types differing in epithelial cell content. It is not known how *CYP1B1* expression varies among different cell types. We suspected that *CYP1B1* expression might be highest in epithelial cells and had planned to correct for epithelial cell content by determining expression of keratin-18, a specific marker of epithelial cells. In addition, we planned to evaluate *CYP1B1* expression levels relative to the  $\beta$ -actin housekeeping gene.

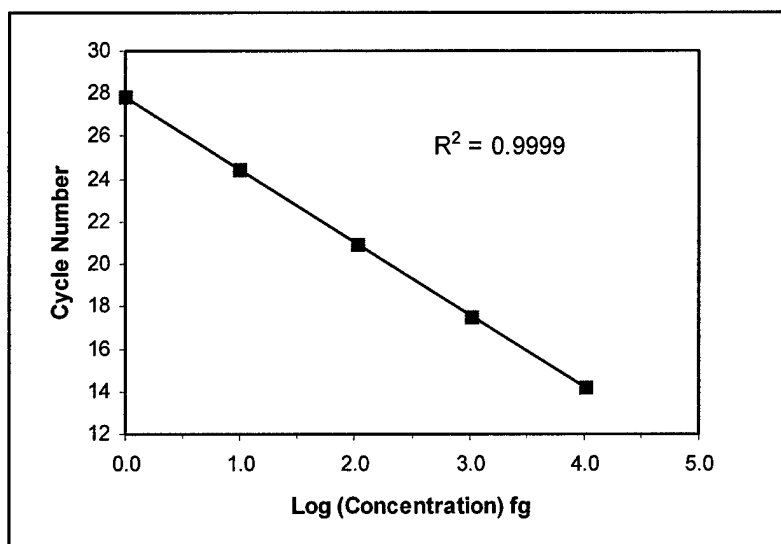
We tested this concept by measuring expression of *CYP1B1*, keratin-18 and  $\beta$ -actin in fractions of an archived breast tissue specimen prepared by stepwise filtration through filters with decreasing pore size from 150, 95 to 51  $\mu$  leaving as a last fraction the filtrate (Stampfer, 1985). Calibrator-normalized RT-PCR was performed with the LightCycler instrument. The specificity of each product was checked by melting curve analysis. The expression level in the sample was corrected by the total amount of RNA added to the reaction and then normalized to our calibrator sample isolated from the HMEC184 cell line. The results are summarized in Table 1. Keratin-18 expression was highest in the tissue retained by the 150  $\mu$  filter and decreased 5-10 fold in the other fractions, indicating that the 150  $\mu$  fraction contains most epithelial cells. *CYP1B1* expression levels showed only minor variation between the tissue fractions (except in the filtrate), whereas  $\beta$ -actin expression varied more among the fractions. Therefore, when expressing *CYP1B1* transcript levels relative to  $\beta$ -actin in different breast tissue fractions, it seems to incorrectly represent *CYP1B1* expression levels, because  $\beta$ -actin expression varies with cell fraction (Table 1). We have concluded that this housekeeping gene is not an appropriate choice for breast tissue specimens. Therefore, measurement of the  $\beta$ -actin gene will not be used in this study and correction for epithelial cell content appears not to be necessary.

Table 1: Comparison of *CYP1B1*, keratin-18 and  $\beta$ -actin expression levels in different fractions of a single breast tissue specimen determined by Calibrator-normalized RT-PCR.

Tissue retained by filter with pore size (micron)	<i>CYP1B1</i>	Keratin-18	$\beta$ -actin	<i>CYP1B1</i> / $\beta$ -actin
150	0.21	0.37	0.35	0.61
95	0.15	0.06	0.10	1.50
51	0.21	0.08	0.21	1.01
filtrate	0.09	0.04	0.07	1.36

Instead of determining *CYP1B1* expression levels relative to a housekeeping gene, we have decided to quantitate the absolute amount of CYP1B1 transcript found in each specimen. We have developed a Quantitative RT-PCR assay for CYP1B1 using the LightCycler. A CYP1B1 quantitation standard was produced by inserting the CYP1B1 PCR fragment into a plasmid and then cloning it into an *E.coli* cell line. Large quantities of the plasmid were obtained by growing up these *E.coli* cells, then isolating the plasmid. The quantitation standard was produced by *in vitro* transcription from the linearized plasmid. The amount of the CYP1B1 quantitation standard was accurately determined by a fluorescent assay. Each run of the LightCycler will include a dilution series of this standard. The standard and the CYP1B1 gene transcript are reverse transcribed and amplified together at equal efficiencies to control for each step of the assay. The CYP1B1 expression level in an unknown sample is determined by extrapolating from a curve produced from the dilution series of the quantitation standard (Figure 1). To control for variations in specimen quantities, an equal amount of total RNA isolated from each specimen will be added to each RT-PCR reaction.

Figure 1. A standard curve of the CYP1B1 Quantitation Standard measured on the LightCycler.



To test this improved assay, we exposed MCF7 cells to benzo[a]pyrene (BaP) to induce CYP1B1 expression. We measured the induction with the Quantitative RT-PCR assay (Table 2).

Table 2: Induction of CYP1B1 in MCF7 cells treated with increasing concentrations of BaP for 24 hours. Expression levels were measured by Quantitative RT-PCR using an external standard and the LightCycler.

Treatment	pg <i>CYP1B1</i> gene equivalent
Control	0.04
0.25 µg/ml BaP	0.26
1 µg/ml BaP	1.23
2.5 µg/ml BaP	1.27

**Task 3 Measure PAH-DNA adducts by  $^{32}\text{P}$ -postlabeling in breast tissue and**

**Task 4 Analyze data and write reports** have not been initiated because of the delay in recruitment. We have requested and obtained an 18 months no-cost extension until 3/06.

### **KEY RESEARCH ACCOMPLISHMENTS**

- Received full IRB approval from Brazilian National Ethics Committee.
- Hired and trained field personnel and set up the laboratory in Porto Alegre.
- Developed, tested and finalized all forms, protocols, questionnaire and data entry system.
- Established monthly conference calls with Brazilian field staff and monthly reports.
- Worked out shipping conditions of specimens and set up contract with World Courier.
- Cloned a CYP1B1 gene fragment to be used as a quantitative standard.
- Measured CYP1B1 expression in different cell fractions of normal breast tissue.
- Enrolled 5 participants.
- Shipped first samples to LBNL 9/28/04.

### **REPORTABLE OUTCOMES**

None

### **CONCLUSIONS**

After long delays, we are now actively collecting data and specimens in the field to test our hypothesis that high *CYP1B1* expression results in high levels of PAH-DNA adducts and increases the risk of breast cancer.

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## **APPENDICES**

Participant Tracking Form  
Protocol for Urine Collection and Transportation  
Protocol for Blood Collection  
Protocol for Epithelial Breast Tissue Collection  
Protocol for Adipose Breast Tissue Collection  
Protocol for Breast Tissue and Blood Transportation  
Protocol for Lymphocyte Separation and Storage  
Protocol for Urine Cell Separation and Storage  
Protocol for Shipping Specimens to LBNL  
Participant Tracking Form  
Shipping Log  
August 04 Tracking Form

**Role of CYP1B1 in PAH-DNA Adduct Formation and Breast Cancer Risk**

**PARTICIPANT TRACKING FORM**

(last updated on 12/1/2003)

**Recruitment**

**Date Completed:** \_\_\_\_\_

- ☐ RECRUITMENT FORM received
- ☐ ELIGIBILITY CRITERIA checked
- ☐ INFORMED CONSENT FORM signed and received
- ☐ Participant file set up & ID# assigned

**Specimen Collection**

**Date Completed :** \_\_\_\_\_

- ☐ Urine specimen received, processed, and stored by LRC
- ☐ Blood specimen received, processed, and stored by LRC
- ☐ Breast epithelial tissue specimen received, processed, and stored by LRC
- ☐ Breast adipose tissue specimen received, processed, and stored by LRC
- ☐ URINE LAB LOG labeled with ID#, completed, and filed
- ☐ BLOOD LAB LOG labeled with ID#, completed, and filed
- ☐ BREAST TISSUE LAB LOG labeled with ID#, completed, and filed
- ☐ Special study pathology report and slide received and filed/stored

**Questionnaire/Interview**

**Date Completed :** \_\_\_\_\_

- ☐ Interview scheduled
- ☐ Interview completed
- ☐ Questionnaire labeled with ID# and checked by Interviewer
- ☐ Questionnaire labeled with ID# and checked by ERC, and filed

**Medical Record Abstraction**

**Date Completed :** \_\_\_\_\_

- ☐ MEDICAL RECORD ABSTRACTION FORM completed and filed
- ☐ Pathology report copied, redacted, labeled with ID#, and filed

**Data Entry**

**Date Completed :** \_\_\_\_\_

- ☐ Questionnaire data entered by Interviewer #1
- ☐ Questionnaire data entered by Interviewer #2
- ☐ Medical record data coded and entered by ERC
- ☐ Medical record data entered by LRC
- ☐ Database QA/QC validation checks completed

**Shipment**

**Date Completed :** \_\_\_\_\_

- ☐ Specimens shipped to LBNL (U \_\_/\_\_, UC \_\_/\_\_, LC \_\_/\_\_, ET \_\_/\_\_, AT \_\_/\_\_)

## Role of CYP1B1 in PAH-DNA Adduct Formation and Breast Cancer Risk

### Protocol for Urine Collection and Transportation

(last updated on 12/2/2003)

This document describes the procedure for collecting urine from study participants and for transporting the urine specimen to the laboratory.

---

#### Supplies Required

sterile specimen cup	ID labels
participant instructions	lab marker
towlettes	cold gel packs
gloves	Urine Lab Log
transport cooler	disinfectant* in wash bottle
paper towels	

\*10% bleach or 70% ethanol to clean up any spills.

#### Before you begin

1. Schedule an appointment with participant.
2. Identify the ID# of the participant and bring the appropriate ID labels.
3. Fill the transport cooler with *cold* gel packs.

#### Procedure

1. Make sure that the Urine Lab Log is stamped with the appropriate ID #.
2. Record the date and your initials in Part 1 of the Urine Lab Log.
3. Record the date of the participant's last day of her last period in Part 1 of the Urine Lab Log.
4. Instruct the participant on how to collect the urine sample. Give the participant the Participant Urine Collection Instructions to read.
5. Give the participant a sterile specimen cup and 2 packages of towlettes.
6. Wear gloves.
7. Retrieve the specimen cup as soon as the participant has finished.
8. Tighten the lid on the specimen cup. Use a towlette to clean the outside of the specimen cup. Let the surface of the cup dry.
9. Apply the pre-numbered ID label to the side of the cup (not the lid).
10. Place the urine sample into the transport cooler so that it remains upright.
11. Discard your gloves.
12. Record the time in the Urine Lab Log.
13. Return the specimen to the laboratory as soon as possible (i.e., same day, within 2-3 hours).
14. Store the urine in the refrigerator.
15. Record time stored in the refrigerator on the Urine Lab Log.

## Role of CYP1B1 in PAH-DNA Adduct Formation and Breast Cancer Risk

### Protocol for Blood Collection

(last updated on 12/1/2003)

This document describes the procedure collecting whole blood in BD vacutainer CPT tubes.

---

#### Supplies Required

BD vacutainer CPT tube  
Blood Lab Log  
gloves  
tube rack

ID labels  
lab marker  
venipuncture supplies

#### Before you begin

1. All work is to be performed in a sterile manner.
2. Follow all biosafety precautions for protection from blood borne pathogens.
3. Identify the ID# of the participant and bring the appropriate ID labels.
4. Prepare supplies listed above.

#### Procedure

1. Make sure that the Blood Lab Log is stamped with the appropriate ID #.
2. Record the date, time, and your initials in Part 1 of the Blood Lab Log.
3. Label one Becton-Dickinson (BD) vacutainer CPT (8 ml size with citrate) with the appropriate ID label.
4. Work with surgical team to collect 7 ml of whole blood. Standard venipuncture techniques should be used to collect whole blood into the labeled BD vacutainer CPT tube at the time of surgery. The same needlestick that used for other surgery-related blood draws should be used as per the human subjects protocol. Be careful to prevent backflow.
5. Store the sample upright in the tube rack at room temperature.
6. Be sure the cap is firmly placed on the BD vacutainer CPT tube. Mix the blood sample by inverting the tube 5 times. **Do not shake.**
7. Begin the lymphocyte separation procedure within 2 hours of collection.

## Role of CYP1B1 in PAH-DNA Adduct Formation and Breast Cancer Risk

### Protocol for Epithelial Breast Tissue Collection

(last updated on 6/9/2004)

This document describes the procedure for preparing and storing non-tumor breast epithelial tissue that would otherwise be discarded during surgery.

---

Breast tissue that would otherwise be discarded during surgery must be collected and prepared for storage as soon as possible. This procedure requires you to identify non-tumor epithelial tissue, to clean the specimen from contaminants, and finally, to preserve only the epithelial material in 5x the volume of *RNA Later*. It is essential for the study that only non-tumor epithelial tissue be preserved. Some epithelial tissue will be sent to the pathologist.

#### Supplies Required

dissection tray	gloves	Breast Tissue Lab Log
scalpel or scissors	15 ml storage tubes	isopropanol**
forceps	tube rack	isopropanol** in wash bottle
ruler	ID labels	small glass beaker (50 ml)
<i>RNA Later</i>	sterile PBS in wash bottle	disinfectant* in wash bottle
10 ml sterile pipet	lab marker	Protocol-Adipose Collection
*10% bleach or 70% ethanol to clean up any spills.		

#### Before you begin

1. All work is to be performed in a sterile and chemically-clean manner.
2. Before surgery, ensure that the dissection tray, scalpel, scissors, ruler forceps are clean and sterile. Autoclave if necessary.
3. Before surgery, clean the dissection tray and instruments with isopropanol as described in the Protocol for Adipose Breast Tissue Collection.
4. **Dispense 10 ml of *RNA Later* into a sterile 15 ml storage tube and cap the tube. Prepare 3 of these tubes.** Use lab marker to label tubes with the date and "*RNA Later*." Store them at room temperature with the caps on until surgery. These may be prepared several days ahead of time.
5. Fill the wash bottle with fresh, sterile PBS (saline) or use sterile water from the surgery if available.
6. Identify the ID# of the participant and bring the appropriate ID labels.
7. Be sure that the Breast Tissue Lab Log is stamped with the appropriate ID #.
8. Record the date, start time, and initials in Part 1 of the Breast Tissue Lab Log.

### **Dissection of epithelial tissue**

1. Collect the discarded tissue from surgery on the chemically-clean dissection tray.
2. Identify the non-tumor epithelial tissue and remove any adipose, tumor or connective tissue by dissection with the chemically-clean scalpel and/or scissors.
3. Separate some epithelial tissue and prepare as per instructions from the pathologist.
4. Send a portion of epithelial tissue for pathologic analysis as per instructions from the pathologist.
5. Rinse the remaining epithelial material with sterile saline (PBS) or sterile water to remove any blood.

### **Preservation of epithelial tissue in RNALater**

1. Preserve as much epithelial tissue as possible by placing it in the storage tubes filled with RNALater. **Only 2 g of tissue can be preserved in 10 ml of RNALater in one tube.** Fill as many tubes as necessary. Estimate the amount of epithelial tissue by the volume of RNALater that is displaced as described below.
2. The epithelial material must be cut to the appropriate size. To ensure proper preservation, the tissue can only be 0.5 cm thick in any one direction. Use the ruler to measure the thickness of the tissue. Cut the tissue so that it is 0.5 cm in any one direction.
3. **Each storage tube containing 10 ml of RNALater can only preserve a total volume of 2 mg of tissue.** Using sterile forceps, place epithelial tissue pieces in one of the storage tubes containing 10 ml of RNALater. **Continue placing tissue pieces into the tube until the volume reaches the 12 ml mark. Be sure to submerge the tissue into the fluid.**
4. Read the volume in the tube. If the volume exceeds 12 ml, then you have placed too much tissue in the tube. Remove the tissue and cut it into smaller pieces. Place each piece separately into another RNALater containing tube. Fill as many tubes as necessary to preserve all of the tissue.
5. Any tubes of RNALater that are not used may be saved for the next surgery.
6. When all the tissue has been placed in storage tubes containing RNALater, cap the tubes tightly. Make sure there are no air bubbles surrounding the tissue. The tissue should be completely covered with RNALater.
7. Label each 15 ml tube with the appropriate ID label.
8. Record the number of tubes prepared in Part 1 of the Breast Tissue Lab Log.
9. Record the end time in Part 1 of the Breast Tissue Lab Log.
10. Transport to laboratory (see Protocol-BreastTissueBloodTransport).
11. Store the specimens preserved in RNALater in the refrigerator for at least 24 hours, but no longer than 3 days.
12. Clean the dissection tray, forceps, scissors, scalpel, and ruler with disinfectant. Autoclave before the next use

### **The Next Day: Freezer Storage**

1. Confirm that the tissue specimen have been soaking in *RNALater* for 24 hours. The tissues are now preserved.
2. **Combine the tissue specimen to only 2 tubes.** (Only 2 tubes will be shipped to LBNL.) If 3 tubes have been filled, use sterile forceps to transfer the tissue specimen from one tube to the other two. At this point, the specimens are preserved and it is OK to overfill the tubes with tissue. Discard the third tube. The used *RNALater* may be dumped down the sink according to your waste procedures.
3. Transfer the breast epithelial tissue specimens preserved in *RNALater* to the freezer. Confirm that the freezer temperature is **no colder than  $-20^{\circ}\text{C}$** . Record the position of the tubes on the freezer map.
4. After moving the specimen to the freezer, record date, time, and your initials in Part 3 of the Breast Tissue Lab Log.

## Role of CYP1B1 in PAH-DNA Adduct Formation and Breast Cancer Risk

### Protocol for Adipose Breast Tissue Storage

(last updated on 5/27/2004)

This document describes the procedure for preparing and storing breast adipose tissue that would otherwise be discarded during surgery.

---

Tissue that would otherwise be discarded during surgery must be collected and prepared for storage as soon as possible. This procedure requires you to identify adipose tissue and to preserve the adipose tissue in glass vials. Two vials will be preserved. **It is important to keep specimens free of chemical contamination. Handle specimen quickly and as little as possible. All instruments and surfaces need to be cleaned with a solvent.**

#### Supplies Required

dissection tray	paper towels or tissue
scalpel or scissors	tube rack
forceps	ID labels
gloves	lab marker
4 ml glass vials, 2 each	Breast Tissue Lab Log
isopropanol**	disinfectant* in wash bottle
small glass beaker (50 ml)	isopropanol** in wash bottle

\*10% bleach or 70% ethanol to clean up any spills.  
\*\*If isopropanol is not available, use 95% ethanol

#### Before you begin

1. All work is to be performed in a clean (chemical-free) manner.
2. Prepare all materials listed above.
3. Before surgery, clean the dissection tray with isopropanol. Use the wash bottle to wet the surface of the tray then wipe it with towels to remove any dust particles.
4. Clean the beaker by rinsing three times with isopropanol from the wash bottle. Discard the washes down the sink. Fill the beaker half way with fresh isopropanol. Always use fresh isopropanol for each dissection.
5. Clean the scalpel, scissors, forceps (and any other instrument) by dipping them into the beaker of isopropanol. Wipe them dry with towels and place them on the cleaned dissection tray. Any part of the instrument that may come into contact with the specimen needs to be cleaned with isopropanol.
6. Identify the ID# of the participant and bring the appropriate ID labels.

#### Procedure for adipose tissue

1. Be sure that the Breast Tissue Lab Log is stamped with the appropriate ID #.
2. Label two glass vials with the appropriate ID#.
3. Collect the discarded tissue from surgery on the chemically-clean dissection tray.
4. Identify the adipose tissue and cut off a section that can fit into the glass vial. Do not rinse or clean the specimen.
5. Using clean forceps, place the adipose tissue into the labeled 4 ml glass vial. Fill 2 glass vials.



6. Cap the glass vials tightly.
7. Discard the isopropanol from the beaker. Do not re-use the isopropanol from the beaker. (Isopropanol in the wash bottle may be re-used.)
8. Clean the dissection tray, forceps, scissors, and scalpel with disinfectant. Autoclave before the next use.
9. Transport to laboratory (see Protocol-BreastTissueBloodTransport).
10. Store the specimens in the freezer. Record position on freezer map.
11. Record date, time, and initials in Part 2 of the Breast Tissue Lab Log.

## **Role of CYP1B1 in PAH-DNA Adduct Formation and Breast Cancer Risk**

### **Protocol for Breast Tissue and Blood Transportation**

(last updated on 12/1/2003)

This document describes the procedure for transporting whole blood and breast tissue (epithelial and adipose) from the surgery to the laboratory.

---

#### **Supplies Required**

gloves	cold gel packs
transport cooler	packing material
tube rack	Blood Lab Log
ID labels	Breast Tissue Lab Log
lab marker	disinfectant* in wash bottle
paper towels	

\*10% bleach or 70% ethanol to clean up any spills.

#### **Before you begin**

1. Place gel packs in the refrigerator 24 hours before surgery.
2. Fill the transport cooler with cold gel packs.

#### **Procedure**

1. Place cold ice packs in the bottom of the transport cooler.
2. Place the tube rack on the cold gel packs.
3. Place the specimens in the tube rack so that they will not tip over (use packing material, such as newspaper to support the tube rack).
4. Add more ice packs along the sides of the tube rack.
5. Fill the cooler with packing material so that the samples will not move around.
6. Place the specimens into the transport cooler so that they remain upright.
7. Seal the cooler.
8. Transport the specimens to the laboratory immediately.
9. Store breast adipose tissue in freezer.
10. Store breast epithelial tissue in refrigerator overnight (refer to Protocol-BreastEpithelialTissueCollection).
11. Record lab time in Part 1 of the Breast Tissue Lab Log.
12. Begin lymphocyte separation immediately and then urine separation (see Protocol-LymphocyteSeparation and Protocol-UrineSeparation).

## Role of CYP1B1 in PAH-DNA Adduct Formation and Breast Cancer Risk Protocol for Lymphocyte Separation and Storage

(last updated on 12/1/2003)

This document describes the procedure for separating lymphocytes from freshly collected whole blood in BD vacutainer CPT tubes. **It is essential to begin the procedure within 2 hours after collection.**

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### Supplies Required

centrifuge	15 ml tubes	10 ml sterile pipets
whole blood	tube rack	15 ml balance tube
BD vacutainer CPT tube	ID labels	disinfectant* in wash bottle
RNA <i>Later</i>	sterile PBS	waste beaker for plasma
sterile transfer pipets	lab marker	biohazard waste basket
Blood Lab Log	paper towels	sterile specimen cup
gloves	vacutainer balance tube	

\*10% bleach or 70% ethanol to clean up any spills.

### Before you begin

1. All work is to be performed in a sterile manner.
2. Follow all biosafety precautions for protection from blood borne pathogens.
3. Identify the ID# of the participant and bring the appropriate ID labels.
4. Prepare supplies and reagents listed above.
5. Label one sterile specimen cup "PBS" and dispense 20 ml PBS into this cup.

### Procedure

1. Obtain the whole blood sample (7 ml) in a BD vacutainer CPT tube from surgery. Store the sample upright at room temperature.
2. Confirm the specimen ID# on the vacutainer.
3. Record date, start time, and your initials in Part 2 of the Blood Lab Log.
4. Begin this lymphocyte separation procedure within 2 hours of collection.
5. Be sure the cap is firmly placed on the vacutainer. Mix the blood sample by inverting the vacutainer 10 times just before centrifugation. **Do not shake.**
6. Adjust the level of PBS in the vacutainer balance tube to the same level as that in the specimen vacutainer.

**NOTE: Take PBS from the specimen cup only! Do not put pipets into the original PBS bottle. This will help prevent contamination.**

7. Place the specimen vacutainer into the centrifuge and balance the centrifuge using the balance vacutainer.
8. Set the centrifuge speed to 43 x 100 rpm (1757 rcf) and spin the samples for 30 minutes at room temperature. Do not exceed this rpm setting as the glass vacutainer may break.
9. Label two sterile 15 ml tubes with the participant ID# using the lab marker.
10. Carefully remove the vacutainer from the centrifuge. The blood will be separated into layers (see Figure 1). The lymphocytes will be a cloudy white layer located just above the gel barrier. Some red blood cell contamination is normal. (See the diagram in the product insert for more information). Collect the lymphocytes immediately after centrifugation.
11. Use a sterile pipet to remove at least 2 to 3 ml of the top plasma layer (yellow) without disturbing the lymphocytes. Discard this plasma.

12. Use the same pipet to resuspend the lymphocytes, then transfer the lymphocytes and the remaining plasma to the prepared 15 ml tube.
13. Discard the vacutainer.
14. **WASH #1:** Use a fresh pipet to add sterile PBS (from specimen cup) to the lymphocytes in the 15 ml tube until the volume reads 10 ml. Cap the tube. Mix by inverting 5 times gently. **Do not shake.** Place the 15 ml tube in the centrifuge and balance with a 15 ml balance tube. (You may need to adjust the volume of PBS in the balance tube).
15. Set the centrifuge speed to 22 x 100 rpm (460 rcf) and spin for 10 minutes.
16. Use a pipet to remove as much PBS as possible without disturbing the lymphocytes. (As much as half the PBS may remain).
17. Gently resuspend the lymphocytes in the remaining PBS and transfer them to a fresh 15 ml tube. Some red blood cells will remain behind. Discard the first 15ml tube.
18. **WASH #2:** Fill the 15 ml tube containing the lymphocytes to 10 ml with sterile PBS (from specimen cup). Invert gently 5 times to mix. Place in the centrifuge and balance.
19. Set the centrifuge speed to 22 x 100 rpm (460 rcf) and spin for 10 minutes.
20. Carefully discard the PBS. Leave no more than 2 ml of PBS.
21. **WASH #3:** Fill the 15 ml tube containing the lymphocytes to 10 ml with sterile PBS (from specimen cup). Invert gently 5 times to mix.
22. Set the centrifuge speed to 22 x 100 rpm (460 rcf) and spin for 10 minutes.
23. Carefully discard the PBS. Leave about 0.5 ml of PBS. The cells may be faintly pink.
24. Gently resuspend the lymphocytes in the remaining PBS.
25. Use a new 10 ml sterile pipet to add 6 ml RNALater to the lymphocytes. **Never place a used pipet into the RNALater bottle.**
26. Invert gently 5 times to disperse the lymphocytes into the RNALater.
27. Label the tube with the appropriate participant ID label. Make sure the specimen ID#s agree.
28. Store the lymphocytes in the refrigerator (about 10°C) overnight.
29. Record end time in Part 2 of the Blood Lab Log.
30. Transfer the specimen to the freezer the next day.
31. Record date, time, and your initials in Part 3 of the Blood Lab Log after moving lymphocytes to freezer.

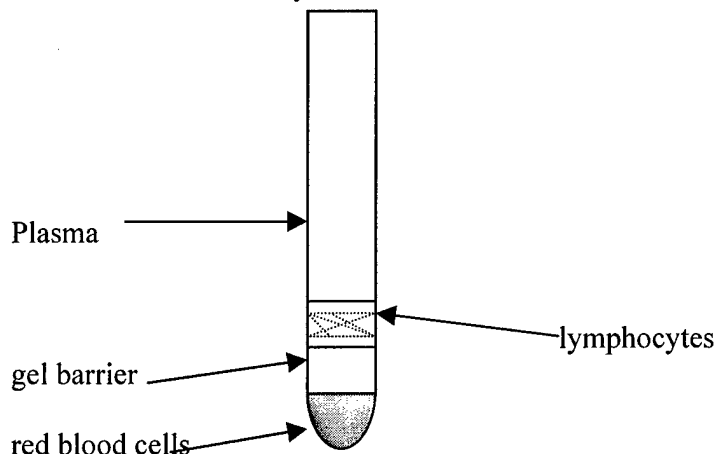


Figure 1. BD vacutainer CPT showing separation of blood components after centrifugation.

## Role of CYP1B1 in PAH-DNA Adduct Formation and Breast Cancer Risk

### Protocol for Urine Cell Separation and Storage

(last updated on 12/2/2003)

This document describes the procedure for collecting exfoliated cells in urine specimens by centrifugation. Both the urine and the cell pellet will be saved.

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#### Supplies Required

centrifuge	tube rack	Urine Lab Log
urine specimen	ID labels	10 ml sterile pipet
<i>RNA later</i>	sterile PBS	15 ml balance tube
sterile transfer pipet	lab marker	disinfectant* in wash bottle
gloves	sterile specimen cup	
15 ml storage tubes	paper towels	
*10% bleach or 70% ethanol to clean up any spills.		

#### Before you begin

1. All work is to be performed in a sterile manner.
2. Identify the ID# of the participant and bring the appropriate ID labels.
3. Prepare supplies and reagents listed above.
4. Label sterile specimen cup "PBS" and add about 20 ml PBS to cup.

#### Procedure

1. Record data, start time, and your initials in Part 2 of the Urine Lab Log.
2. Begin this separation procedure within 24 hours of specimen collection.
3. Estimate the volume of urine collected. Record the volume on the Urine Lab Log.
4. Label the appropriate number of 15 ml storage tubes with lab marker. (A full specimen cup requires 8 storage tubes each containing 14 ml of urine).
5. Use a new 10 ml sterile pipet to transfer the urine to 15 ml storage tubes. Be sure each tube receives the same volume of urine. Prepare a balance tube if you have an odd number of tubes.
6. Place the urine specimens into the centrifuge and balance the centrifuge. Only 4 of the tubes can be centrifuged at once.
7. Spin the samples at 30 x 100 rpm (855 rcf) for 15 minutes at room temperature.
8. Prepare the appropriate number of 15 ml storage tubes for the urine fluid. Label the tubes with the ID labels.
9. Carefully remove the tubes from the centrifuge and place in a tube rack. A white cell pellet should be visible.
10. Use a 10 ml sterile pipet to transfer the urine into the new, labeled 15 ml storage tubes. Place the cap on tightly and store in the freezer.
11. Use a new sterile pipet to add about 1 ml sterile PBS to the cell pellet in each original tube. Resuspend each cell pellet. Use the same pipet to combine the cells into one tube. All the cells should be combined into one tube. Discard the empty, used tubes.

**NOTE: Take PBS from sterile specimen cup only. Do not place used pipets in original PBS bottle. This will prevent contamination.**

12. WASH #1: Using a new sterile transfer pipet, add PBS to the cells until the volume reads 10 ml. Cap the tube. Mix by inverting 5 times gently.
13. Place the tube in the centrifuge and balance.
14. Spin the sample at 30 x 100 rpm (855 rcf) for 10 minutes at room temperature.
15. Use a pipet to discard as much PBS as possible. The cell pellet may be translucent and difficult to see. (OK to use same pipet for this step).
16. WASH #2: Using a new sterile transfer pipet, add PBS to the cells until the volume reads 10 ml. Invert gently 5 times to mix. (NOTE: Take PBS from specimen cup).
17. Spin the samples at 30 x 100 rpm (855 rcf) for 10 minutes at room temperature.
18. Carefully discard the PBS. (OK to use same pipet for this step). Leave only 0.5 ml PBS in the tube. (Add some back if necessary.)
19. Gently resuspend the cells in the remaining (0.5ml) PBS.
20. Using a new 10 ml sterile pipet, add 6 ml RNALater to the cell pellet.
21. **NOTE: Never place a used pipet in the original reagent bottle.**
22. Invert gently 5 times to disperse the cells into the RNALater.
23. Place an ID label on the tube.
24. Store the cell pellet in the refrigerator (about 10°C) overnight.
25. Record end time in Part 2 of Urine Lab Log. Complete Part 2 of the Urine Lab Log.
26. Transfer the urine cell specimen the freezer the next day.
27. After moving specimen to freezer, record date, time, and initials in Part 3 on Urine Lab Log.

## **Role of CYP1B1 in PAH-DNA Adduct Formation and Breast Cancer Risk**

### **Protocol for Shipping Specimens to LBNL via World Courier**

(last updated on 6/08/2004)

This document describes the procedure for transporting only epithelial specimens (ET), lymphocytes (LC), and urinary tract cells (UC) from the laboratory in Brazil to Lawrence Berkeley National Laboratory in California. Do not send urine or adipose specimens at this time. Specimens are to be shipped frozen (at 0° C) using World Courier.

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#### **World Courier Contact Information**

Account number: 10508 (valid 4/2/2004 to 9/1/2005)

Sau Paulo office: 55-11-5591-6262

Internet site:

[www.worldcourier.com/br](http://www.worldcourier.com/br)

U.S. customer service: (800) 221-6600

Internet site:

[www.worldcourier.com/us](http://www.worldcourier.com/us)

#### **Schedule**

Packages will be shipped when specimens from 20 participants are accumulated. (Except for the very first shipment: specimens from 10 participants will be shipped.)

1. Coordinate shipping date with Marion Russell or Regine Goth-Goldstein at LBNL (Tel: 510-495-2915, Email: [mlrussell@lbl.gov](mailto:mlrussell@lbl.gov) or [R\\_Goth-Goldstein@lbl.gov](mailto:R_Goth-Goldstein@lbl.gov)). Confirm that someone will be available to receive the shipment on the day that it will arrive. Never ship on a Friday, Saturday, or the day before a holiday.
2. Coordinate shipping date and time with World Courier to get special "same-day or next flight-out" service. (World Courier will know the flight schedule from Porto Alegre to the United States.) Arrange to have the courier come to the laboratory at Hospital Santa Rita.

#### **Supplies Required**

Specimens (ET, LC, & UC)  
sealing tape (for tubes)  
zip lock bags  
absorbent material  
packing material

frozen gel packs  
transport cooler  
corrugated box  
packing tape  
Shipping Log

Shipping documentation  
CDC permit  
CDC shipping labels  
pen

#### **Before you begin**

1. Most of the time, packages will contain specimen from 20 participants. There will be a total of 80 tubes. For each participant you will ship:
  - 2 Epithelial specimen (labeled EC)
  - 1 Lymphocyte specimen (labeled LC)
  - 1 Urinary tract cell specimen (labeled UC)
2. Contact World Courier to order shipping boxes and gel packs. Talk to World Courier to determine the size and number of boxes necessary, and the number of gel packs required to keep the specimens frozen.
3. Place gel packs in the freezer at least 24 hours before they are needed.
4. Fill out shipping log and find all the specimens that will be shipped. Take an inventory of the specimens.

5. Be sure the lid of every specimen tube is securely fastened.
6. Review instructions for the safe shipment of biological materials. (File "UNH-Shipping-Biological-Materials.pdf" is located on laptop computer.)

**Prepare Shipping Documentation (Please see attached examples.)**

Work with the World Courier office in Brazil to prepare the necessary shipping documentation and labels. This is necessary for customs pre-clearance. Here are some of the forms needed:

1. Customs Invoice (must be on Hospital or Clinic letterhead paper) must include the following:
  - Human specimens are for research purposes only
  - Specimen are not for resale
  - Specimen have no commercial value
  - Assign a value for customs purposes (\$10 US)
  - Total number of specimen, amount of each specimen
2. Declaration to USDA (must be on Hospital or Clinic letterhead paper) must include the following:
  - Human tissue, lymphocytes, and uro-epithelial cells, not infectious
  - Total number of specimen, amount of each specimen
  - Specimen are for lab testing only
  - Specimen are for cancer research only
  - Human material containing no animal material and not of tissue culture origin.
  - Specimens are obtained directly from humans, not recombinant, not cultured.
  - The material was not inoculated with or exposed to infectious agents of agricultural concern including zoonotic agents.
3. CDC permit
4. CDC shipping labels

**Procedure**

1. Organize the shipping documents as necessary according to World Courier directions.
2. Be sure the lids are securely tightened on every sample. Wrap sealing tape (e.g., white lab tape) around the lid of each tube.
3. Place specimens in secondary containers (zip lock bags) with absorbent material and store in the freezer (at  $-20^{\circ}\text{C}$ ) overnight.
4. One or two hours before the courier arrives, finish preparing the package. Place the secondary container (zip lock bags) in the cooler and surround with gel packs as directed by World Courier.
5. Fill any empty space with packing material so that secondary container and gel packs do not move.
6. Seal the cooler with packing tape. Place the cooler in the corrugated box.
7. Place our Shipping Logs into a plastic zip lock bag and seal. Place the bagged shipping logs in the box.
8. Close the box and seal with packing tape.
9. Label the box with the sender's and recipient's addresses using a CDC label:

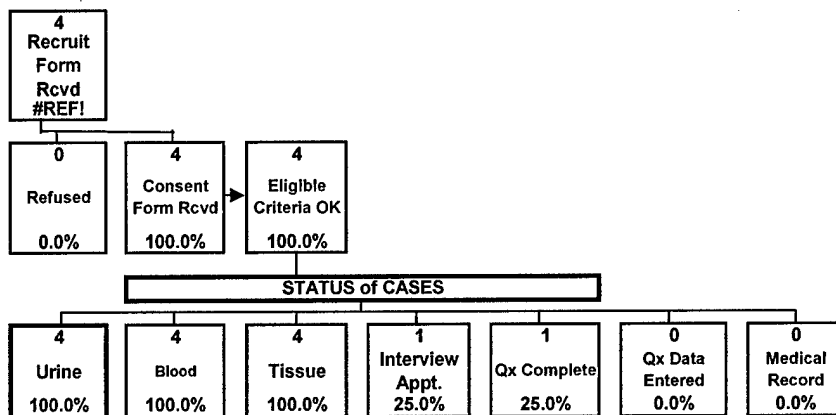
(Consignee)

SHIP TO: Lawrence Berkeley National Laboratory  
Attn: Regine Goth-Goldstein Bldg. 70-264, Tel: 510-495-2915  
One Cyclotron Road  
Berkeley, CA 94720  
U.S.A.



**Role of CYP1B1 in PAH-DNA Adduct Formation and Breast Cancer Risk**  
**MASTER TRACKING FORM - Aug 1 - Aug 31, 2004**

**CASES**



**CONTROLS**

